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14. ABSTRACT

A summary is presented of research performed during three years of a project to determine feasibility of approaches to restore wild-type transcriptional activity on mutant p53 proteins found in human prostate tumors. p53 mutant proteins that are specifically relevant to prostate cancer were examined to determine whether they are suitable targets for such an approach. Three specific aims were pursued. The first was characterizing the interaction of p53 with two distinct classes of its response elements. The second aim was determining the role of mutant p53 proteins in prostate cancer cell proliferation. The final aim was to explore approaches to restore wild-type function to mutant p53 proteins found in prostate cancer. The long-term goals of this research were to identify small molecular weight compounds that have the novel activity of restoring wild-type function to prostate cancer-derived mutant p53 proteins. As such, this represented a preclinical development of highly targeted therapy with the hope of establishing highly effective and tumor-specific treatments of human prostate cancer.

15. SUBJECT TERMS
p53, DNA binding, transcription, gene expression

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Introduction

Genetic alterations of the gene encoding the tumor suppressor p53 are commonly found in a variety of human cancers. Prostate cancer has a 25-30% mutation rate in p53, with a higher prevalence of mutant p53 expression found in late stage or metastatic disease. The challenge of effective cancer chemotherapy is the design of regimens that target tumor cells with minimal side effects on normal cells. Expression of a mutant p53 protein is a characteristic of transformed cells that distinguished them from normal cells. p53 has a well-characterized role in mediating the cellular response to DNA damage. As many chemotherapeutic agents act in a genotoxic manner, it has been proposed that p53 status may be an important determinant for the clinical responsiveness of tumors to treatment with such drugs. Unfortunately, studies have failed to confirm this promising possibility. p53 can trigger either a cell cycle arrest or an apoptotic response depending upon cell type and particular conditions. It has generally been accepted that loss of p53 in tumor cells allows them to evade this surveillance mechanism and become resistant to treatment. Thus, the p53 pathway has been considered a means by which cells respond to genotoxic stress and are eliminated.

The notion that mutant p53 proteins can regain wild-type function was the focus of the research funded by this IDEA award from the Department of Defense Prostate Cancer Research Program. Previous studies from our laboratory demonstrated that genomic binding sites for p53 can be classified into two classes (Resnick-Silverman et al., 1998; Thornborrow and Manfredi, 1999; St. Clair et al., 2004). As tumor-derived p53 mutant proteins have typically lost the ability to bind to DNA in a sequence-specific manner, approaches to restore wild-type function will require a detailed understanding of the molecular basis for DNA binding by p53. Thus, the goals of this research were to characterize the two distinct classes of p53 binding sites, to perform proof-of-principle studies for restoration of wild-type function to mutant p53, and to explore approaches to restore wild-type function that may have therapeutic utility. This report covers the full three-year funding period.

Three specific aims were pursued. The first was characterizing the interaction of p53 with two distinct classes of its response elements. The focus of this aim was a full understanding of the nature of these two subsets of p53 response elements and elucidation of the molecular details of the interaction of p53 with different kinds of binding sites. Such knowledge will be critical for pursuing approaches that restore activity to mutant p53 proteins. The second aim was determining the role of mutant p53 proteins in prostate cancer cell proliferation. Approaches to abrogate mutant p53 expression and to express mutant p53 proteins in both tumorigenic and normal prostate cells were used to determine the role of mutant p53 protein in cell proliferation. "Proof-of-principle" experiments were performed to show the feasibility and usefulness of restoring wild-type activity in mutant p53-expressing prostate tumor cells. The final aim was to explore approaches to restore wild-type function to mutant p53 proteins found in prostate cancer. As the specific mutations found in prostate cancer are distinct from the well-studied "hot spot" mutants, as part of this aim, the activity of these various mutant p53 proteins were examined.

Body

Task 1. Validate the existence of different classes of p53 response elements (Months 1-12)

Seventeen different p53 response elements were placed upstream of a heterologous promoter (that of the minimal adenovirus E1B) driving luciferase expression and were compared in transient transfection assays. In parallel, the same DNA sequences were used in electrophoretic mobility shift assays to compare the relative binding affinity for p53. Although there was a general correlation between in vitro DNA binding and transcriptional activation in the luciferase assay, a subset of sites showed robust binding by p53 in vitro but only minimal activation in the cell-based assay. Two of these elements, the downstream site from the p21 promoter (p21 3') and the upstream site from the PIG3 promoter (PIG3 5') were examined more closely in comparison to two other sites, that from the 14-3-3sigma gene and from the cyclin G1 promoter. These results are shown in Figure 1. While the p21 3' and the 14-3-3sigma sites bound to p53 in a similar manner in vitro, but the 14-3-3sigma site conferred robust p53-dependent transcriptional activation whereas the p21 3' site did not. Similarly, the cyclin G and PIG3 sites showed comparable binding to p53, but only the cyclin G site behaved as a strong p53 response element in this assay. These results validate the existence of two classes of p53 sites which are bound in a comparable manner to p53 but differ in their ability to act as response elements. Previous studies have shown that a monoclonal antibody that binds at the C-terminus of p53 enhances binding to one set of sites and inhibits binding to the other set. It had been previously suggested that the binding of the antibody mimics post-translational modification of p53 at the C-terminus, notable acetylation. Similar results have been obtained by examining mutant p53 proteins that have substitutions at lysines in the

C-terminus that have been implicated as sites of acetylation. Mutation to arginine (conserving charge) does not confer differential binding to the two classes of sites whereas substitution with glutamine (which has been suggested to mimic constitutive acetylation) does cause the mutant p53 to bind with distinct affinity to the different sites (Figure 2). Finally, the ability of p53 to bind to oligonucleotides of varying lengths was examined in in vitro DNA binding assays. The binding of p53 to the p21 5' site appears to depend upon the length of the oligonucleotide in an electrophoretic mobility shift assay, whereas the p21 3' site binding is unaffected (Figure 3). Thus, multiple assays suggest that p53 can interact with different classes of p53 sites in a distinct manner, most likely by adopting a different conformation that is determined by the DNA sequence.

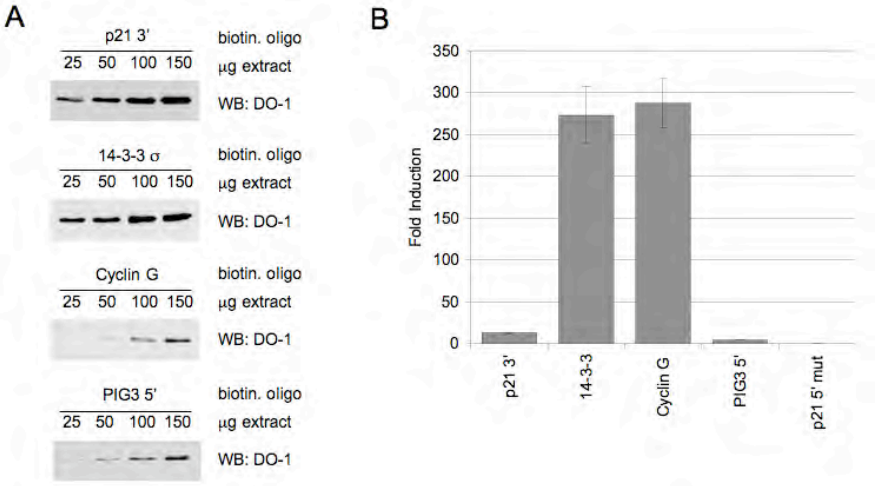

Figure 1: DNA affinity of p53 cells does not correlate with transcriptional activation for a subset of response elements. (A) 25, 50, 100 and 150 mg of extracts were incubated with the indicated biotinylated oligonucleotides. DNA-protein complexes were precipitated with streptavidin beads and the presence of p53 was assayed by Western blot. (B) Cells were transfected with 1 mg of the indicated luciferase reporter constructs and 50 ng of pRL-Renilla. 24 hrs post transfections p53 was induced by removal of tetracyline. Cells were lysed and assayed for luciferase and Renilla activities 24 hrs after induction of p53. The indicated values are the average of three independent experiments, each performed in duplicates.

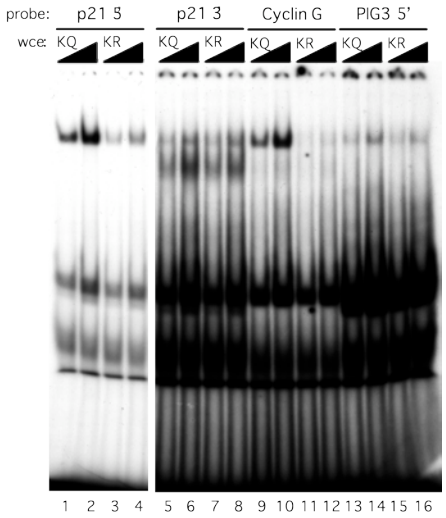

Figure 2. C-terminal mutation affects binding of p53 to a subset of p53 response elements. EMSAs were performed incubating probes containing the p21 5' (lanes 1-4), p21 3' (lanes 5-8), cyclin G (lanes 9-12) or PIG3 5' (lanes 13-16) p53 response elements with two levels of extracts from cells transfected with pCMV-p53^{KQ} or –p53^{KR} mutants.

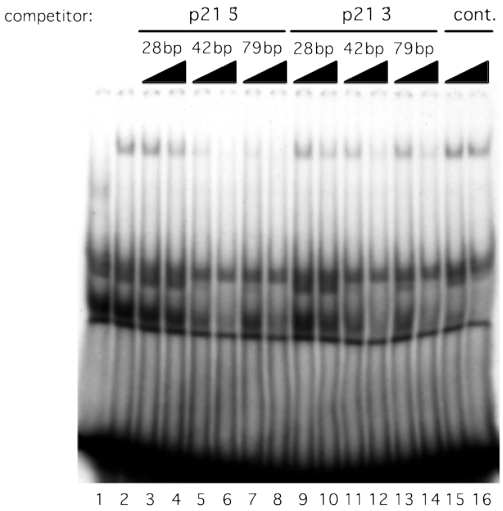

Figure 3. Length of DNA oligonucleotide influences binding of p53 to a subset of response elements. EMSAs were performed incubating the 42-bp radiolabeled p21 5' oligonucleotide with extract from p53-transfected H1299 cells in the presence of mAb 1801 (lanes 2-16). Binding of p53 to the probe was competed with 30- (lanes 3, 5, 7, 9, 11, 13 and 15) or 120-fold (lanes 4, 6, 8, 10, 12, 14 and 16) molar excess of different length unlabeled oligonucleotides containing the p21 5' or 3' response

elements as indicated.

Chromatin immunoprecipation (CHIP) studies have confirmed that p53 can occupy a subset of sites with similar affinity in cells after treatment with DNA damaging agents (Figure 4). This validates the findings using in vitro DNA binding assays. Thus, p53 can interact with a subset of its binding sites to a similar extent and yet its ability to induce transcriptional activation varies. Consistent with the notion that p53 adopts distinct conformations on different binding sites, further experiments have shown that the C-terminus of p53 can regulate binding to the set of sites which are robustly activated, but is dispensable for binding to sites that are weakly activated. Taken together, these studies have indeed validated the existence of different classes of p53 response elements.

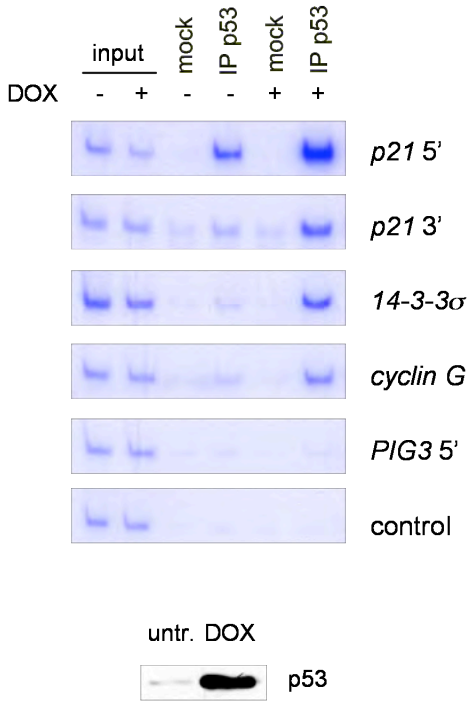

Figure 4. p53 occupies its response elements to similar extent after DNA damage. (A) Cells were treated with 0.5 μg/ml doxorubicin for 12 h. Chromatin immunoprecipitation assays were performed to detect the association of p53 to the response elements in several target genes and a control unrelated region. (B) p53 and actin protein levels in the extracts were assayed by immunoblotting.

Task 2. Determine whether p53 adopts distinct conformations on subsets of its response elements (Months 6-15)

Conditions were established for limited chymotrypsin proteolysis of purified p53 protein in the presence of radiolabeled oligonucleotides containing either the p21 5' site or the p21 3' site. These mixtures were then subjected to electrophoretic mobility shift analysis. Limited proteolytic digestion lead to enhanced binding of p53 to the p21 5' site whereas in the presence of the p21 3' site binding was substantially reduced (Figure 5). However, the effects that were seen were similar whether the DNA was present during the proteolysis or added after the proteolysis reactions were terminated. This indicates that this approach will not be useful for determining whether p53 adopts distinct conformations on the different sites. Nevertheless, these results suggest that limited proteolysis of p53 generates fragments with differing affinity for subsets of binding sites. Further analyses demonstrated that indeed the fragment that shows enhanced binding to the p21 5' site and decreased binding to the p21 3' site lacks the C-terminus. This finding was validated with several other p53 sites representing each class of response element. Use of a mutant p53 that lacks the last 30 amino acids confirmed that similar results can be obtained. Thus, whether the C-terminus is removed by limited proteolysis of through genetic means, the findings were similar and confirm the results obtained with the monoclonal antibody and C-terminal lysine mutants as described above.

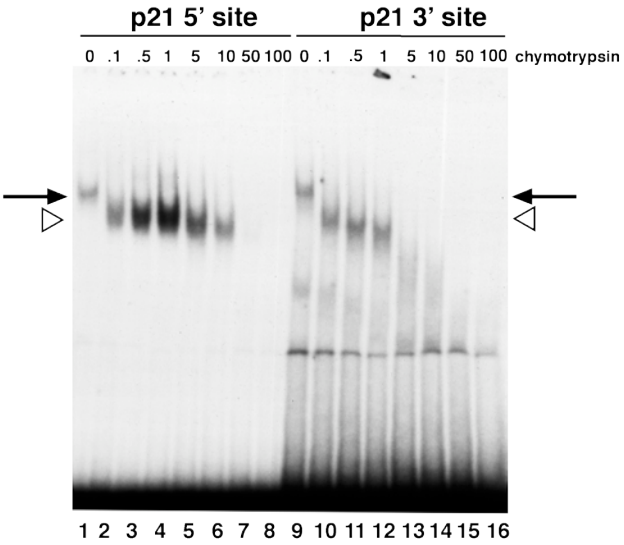

Figure 5. Digestion with chymotrypsin produces truncated forms of p53 that have selectively enhanced binding to the p21 5' site as compared to the p21 3' site. Electrophoretic mobility shift assays were performed using oligonucleotides corresponding to either the p21 5' site or p21 3' site as radiolabeled probe. 10 ng of purified p53 was digested with increasing amounts of chymotrypsin (0-100 ng) for 20 minutes and EMSA was performed in the presence of 500 ng of non-specific poly (dI/dC) competitor DNA.

It was previously reported that conditions for controlled proteolysis of p53 had been established. Effects of different DNA binding sites on proteolysis of p53 were then examined (Figure 6). Each of the sites in the p21 promoter is representative of a distinct subset of p53 response elements. The effect of the mAb 421 on the in vitro DNA binding of p53 to several of its known response elements was tested. These included sites in the promoters of the cyclin G, bax, and cdc25C genes as well as a novel site identified in the first intron of the human p21 gene. This latter site can be shown to bind specifically to p53 in vitro and also confer p53-dependent transcriptional activation on a luciferase reporter containing a minimal adenoviral E1b promoter. The p21 5', p21 intron, and cyclin G binding sites all showed enhanced binding to p53 in the presence of mAb 421, whereas the p21 3', cdc25C, and bax sites did not (Figure 6A). As a control, the mAb 1801 did not alter the binding affinity of p53 for any of the sites. The binding affinities of various sites for the chymotrypsin fragment gave similar results as incubation with mAb 421. The partially digested p53 binding patterns of the cdc25C and bax sites were similar to that of the p21 3' site, showing decreased binding with increased digestion as compared with the enhanced binding to the p21 5' site at the 1-5 ng chymotrypsin level (Figure 6B). However, the p21 intron and cyclin G binding patterns were similar to the p21 5' site with enhanced binding to the partially digested p53. Thus, the p21 5' site is representative of p53 binding sites that show enhanced binding with mAb 421 or partial chymotrypsin digestion. In contrast, the p21 3' site is representative of p53 binding sites in which neither the mAb 421 nor the chymotrypsin fragment confers enhanced binding. These findings form the basis for a revised manuscript that has been resubmitted to J. Mol. Biol. (Maurer et al.).

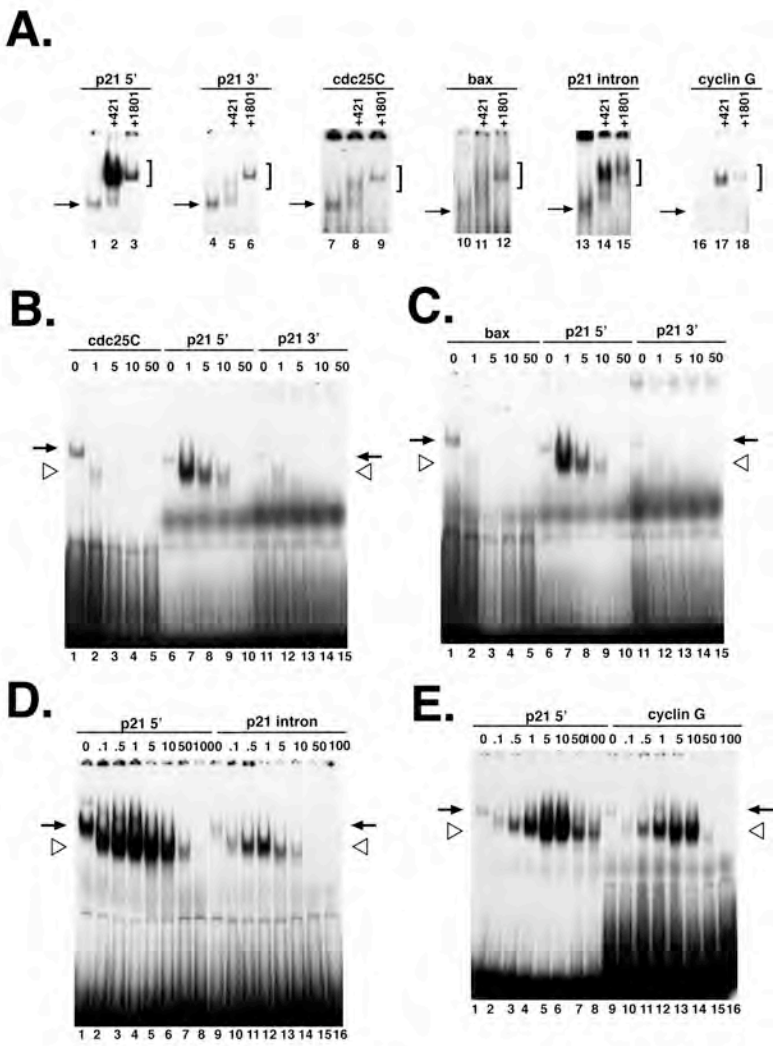

Figure 6. Each of the sites in the p21 promoter is representative of a distinct subset of p53 response elements. (A) EMSA were performed using oligonucleotides corresponding to either the p21 5' site (lanes 1-3), the p21 3' site (lanes 4-6), the cdc25C site (lanes 7-9), the bax site (lanes 10-12), the p21 intronic site (lanes 13-15), or the cyclin G promoter site (lanes 16-18) as radiolabeled probe. 10 ng of purified p53 was incubated with no additions (lanes 1, 4, 7, 10, 13, 16), or in the presence of 800 ng of mAb 1801 (lanes 2, 5, 8, 11, 14, 17), or mAb 421 (lanes 3, 6, 9, 12, 15, 18). The arrows to the left indicate the positions of the p53-DNA complex and the brackets to the right show the super-shifted complex containing antibody, p53, and DNA. (B-E) 10 ng of purified p53 was digested with increasing amounts of chymotrypsin (0-100 ng, as indicated) for 20 minutes and processed as described in Experimental Procedures. EMSA was performed with the indicated sites as radiolabeled probes. For each autoradiogram, the black arrows indicate the position of the DNA complex with intact p53 and the open arrowheads mark the position of the DNA complex containing chymotrypsin-digested p53.

Task 3. Determine the significance of distinct classes of p53 response elements (Months 16-27)

Conditions have been established to detect an interaction between p53 and either of its coactivators CBP or p300 in solution (Figure 7). Studies were performed to examine the ability of p53 to interact with candidate co-factors such as these in pull-down assays using examples of each class of p53 response element and to explore the identities of novel proteins that interact with p53 in a binding site-dependent manner. These studies have been inconclusive to date.

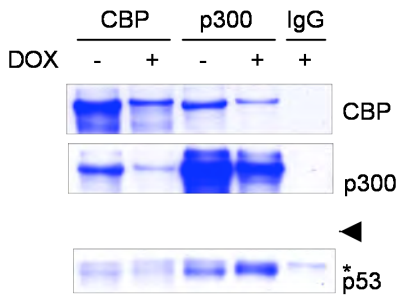

Figure 7. The interaction of p53 with the co-activators CBP and p300 is not affected by DNA damage. Cells were treated with 0.1µg/ml doxorubicin for 24 hr. Cell lysates were then subjected to immunoprecipitation with either anti-CBP, anti-p300, or a control IgG. Immunoblotting for CBP, p300, and p53 was then performed. The asterisk indicates a non-specific band that is detected in all lanes.

Task 4. Determine the role of p53 in mutant p53-expressing DU145 prostate cancer cells (Months 1-27)

An siRNA approach to ablate expression of mutant p53 in the DU145 cell line will be used to determine whether mutant p53 expression is necessary for DU145 cell proliferation. Although it was proposed to use a stable shRNA method, as these studies were being performed, the concern arose that if mutant p53 is required

for proliferation, it may be difficult to determine this using stable transfection. Thus, conditions for transient downregulation of mutant p53 using transfection with siRNA oligonucleotides were explored. The high level of mutant p53 expression in DU145 cells as made this a more difficult task than anticipated. Although methods to ablate p53 in wild-type p53 expression have been quite successful in the laboratory, the level of mutant p53 in DU145 is approximately 10-fold higher than that seen in the wild-type p53 expressing cells. The original approach had been to use a single siRNA oligonucleotide. Although this worked well in the wild-type p53 cells, it was ineffective in DU145 cells. Thus, it was determined that a mixture of four different oligonucleotides was needed to successful downregulate mutant p53 expression in DU145 cells by greater than 95% (Figure 8A). Substantial reduction in mutant p53 expression did not affect the proliferation of these cells as determined by flow cytometric analysis (Figure 8A). DU145 cells were then co-transfected with a plasmid expressing a short hairpin RNA against p53 as well as one that confers resistance to puromycin. Drug-resistant colonies were isolated three weeks later and analyzed for mutant p53 expression (Figure 8B). Clones that showed loss of p53 expression were readily detected. The levels of p53 in six such clones are shown in Figure 8B. No effect on cell proliferation was detected when these clones (D6-D10) were compared to control clones (D1-D5). A representative example is shown in Figure 8B for clone D6. These results suggest that mutant p53 expression is not required for DU145 prostate cell proliferation.

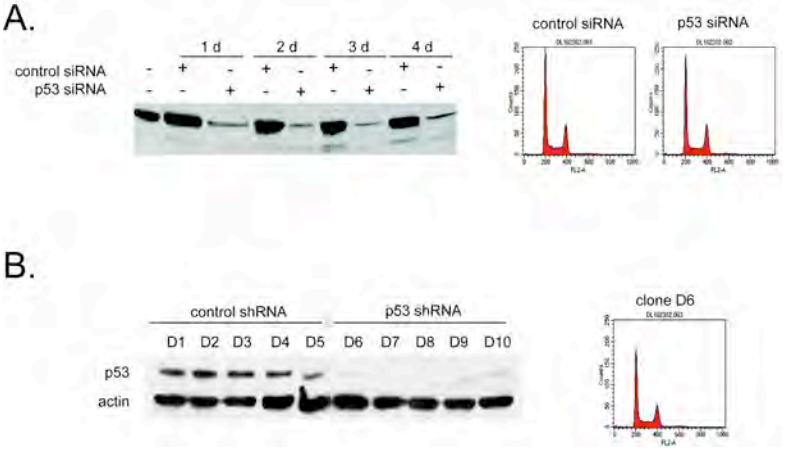

Figure 8. Ablation of p53 expression does not affect proliferation of DU145 prostate cancer cells. (A) DU145 cells were transiently transfected with siRNA oligonucleotides against p53. Immunoblotting for p53 was performed at 1-4 days after transfection (left panel). Cells at day 4 were stained with propidium iodide and subjected to flow cytometric analysis (right panel). (B) DU145 cells were transfected with a plasmid expressing an shRNA against p53 as well as one conferring puromycin resistance. Drug-resistant colonies were isolated and subjected to immunoblotting for p53 (left panel). One such clone (D6) was analyzed by flow cytometry (right panel).

Task 5. Examine the role of prostate tumor-derived mutant p53 proteins in regulating cell proliferation (Months 15-31)

PC-3 cells were transfected with expression plasmids expressing wild-type p53 or the mutants P223L or V274F. These latter two mutants are the p53 proteins which are expressed in the DU145 cell line. These plasmids were co-transfected with one that confers puromycin resistance and colony assays were performed. Wild-type p53 completely suppressed colony formation in this assay whereas the two mutants showed impaired ability to do so (Figure 9A). Levels of expression of the various p53 proteins were shown to be comparable by immunoblotting (Figure 9B). Interestingly, the P223L mutant had comparable activity as wild-type in activating transcription of a luciferase reporter containing the human p21 promoter. In contrast, V274F failed to do so (Figure 9C).

Task 6. Characterize previously identified methods to restore wild-type function on prostate tumor-derived mutant p53 proteins (Months 24-36)

Previous studies have indicated that the ability of mutant p53 to bind to DNA in a sequence-specific manner can be facilitated by incubation with a monoclonal antibody, mAb 421, as well as with a small peptide derived from the C-terminal end of wild-type p53. Various mutant p53 proteins were expressed ectopically in the p53-null PC-3 cells. Extracts were then used in DNA binding assays. In spite of the published data, we were unable to confer DNA binding on mutant p53 proteins using these approaches. It is unclear why such a discrepancy occurred, but may be related to the fact that the specific mutants that were examined in our assays were distinct from those used in the published studies.

Task 7. Establish approaches to screen for compounds which restore the ability of prostate tumor-derived mutant p53 proteins to activate transcription (Months 1-36)

Four p21 reporter constructs were generated as shown in Figure 10. These included the full-length p21 promoter, one that lacks the upstream 5' site, one that lacks the downstream 3' site, and a construct that lacks

both sites. The activity of these reporters was confirmed in transient transfection assays as shown in Figure 10. The luciferase cDNA was then replaced with a cDNA encoding green fluorescent protein (GFP) and the activity of these reporters were then examined in similar transient transfection assays. It was confirmed that GFP expression was obtained in response to co-transfection with wild-type p53 and that each of the mutant reporters showed less activation with the construct lacking both sites showing minimal GFP expression. Attempts were made to establish stable cell lines expressing each of these reporters and to characterize copy number and activity as the next step in establishing a high-through put approach to screen compounds for the ability to restore wild-type activity to mutant p53 proteins.

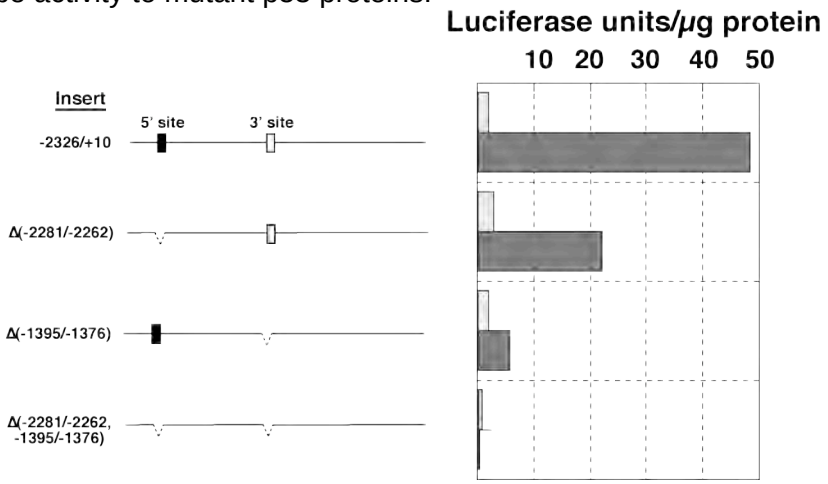

Figure 10. Both the 5' and 3' sites are required for p53-dependent transcriptional activation. The indicated luciferase reporters were generated and co-transfected into p53-null cells with an expression plasmid expressing wild-type p53. The average of three independent experiments performed in duplicate is shown.

Key Research Accomplishments

- Generated luciferase reporters containing 17 different p53 response element
- Generated expression plasmids for C-terminal p53 mutants as well as two prostate-specific tumor derived p53 mutants
- Generated reporters which express green fluorescent protein (GFP) under control of the p21 promoter as well as p21 promoters which lack either the upstream p53 site (p21 5'), the downstream p53 site (p21 3') or both
- Validated the existence of two distinct classes of p53 response elements that are differentially regulating by the C-terminus of p53
- Showed that DNA binding affinity is not the major determinant for transcriptional activation of a subset of targets p53 implicating a role for conformation
- Established methods for limited proteolysis of p53 and used this approach to validate the notion that p53 adopts different conformations on subsets of its genomic binding sites
- Determined that expression of mutant p53 is not required for ongoing proliferation of DU145 cells
- Demonstrated that the mutant proteins expressed by DU145 cells, P223L and V274F, are hypomorphic alleles in that they have impaired activity but do retain some ability to inhibit cell proliferation

Reportable Outcomes

- Maurer, M., Resnick-Silverman, L., Thornborrow, E., and Manfredi, J.J.. The C-terminal region of p53 selectively regulates binding of p53 to a distinct subset of its genomic response elements. J. Mol. Biol, in revision.
- Giono, L.E., Resnick-Silverman, L., Lufkin, D.J., and Manfredi, J.J. DNA binding affinity is not the major determinant for transcriptional activation of a subset of targets by the tumor suppressor p53: a role for conformation and the C-terminus. Submitted.
- Beck, D., Liu, W., Resnick-Silverman, L., and Manfredi, J.J. Two hypo orphic mutants of the p53 tumor suppressor synergize to abrogate the DNA damage response in prostate cancer cells. In preparation.

- Resnick-Silverman, L., Beck, D., Liu, W., and Manfredi, J.J. The tumor suppressor p53 prevents apoptosis in response to chemotherapeutic agents in prostate cancer cells. In preparation.
- Established clones of DU145 cells which lack expression of endogenous p53 protein
- Established clones of PC3 cells which express mutant p53 proteins derived from human prostate tumors

Conclusions

The long-term goals of this research were to identify small molecular weight compounds that have the novel activity of restoring wild-type function to prostate cancer-derived mutant p53 proteins. As such, this represents a preclinical development of highly targeted therapy with the hope of establishing highly effective and tumor-specific treatments of human prostate cancer. Much of the specific research that was performed was laboratory-based and focused on feasibility of such an approach. Nevertheless, it represents necessary preliminary studies that will allow further development and translation of these findings in the future with the ultimate goal of establishing a highly effective and targeted therapy for human prostate carcinoma. As part of these studies, important insights into mutant p53 biology as it relates to human prostate cancer were also gained.

The goal of this research was to explore the role of mutated p53 in prostate tumor cell proliferation. As part of these studies, it was found that prostate tumor cells that lack functional p53 display aberrant cell cycle checkpoints at early time points. This was not unexpected as wild-type p53 has a well-characterized role in mediating the cellular response to genotoxic stress. In related studies, not directly proposed in the grant application, surprisingly, long-term treatment of such prostate tumor cells lacking p53 activity resulted in a cell death response consistent with apoptosis. This suggested that, in fact, p53 plays a role in enhancing prostate tumor cell survival in particular settings. Detailed analyses revealed that wild-type p53 prostate tumor cells respond to sustained treatments with a prolonged growth arrest with the hallmarks of senescence. These same cells, however, will fully recover from transient treatments due to the reversibility of the p53-dependent checkpoint. In contrast, prostate tumor cells lacking a functional p53 fail to maintain the checkpoint, enter mitosis without repairing the DNA damage, and subsequently sustain death by apoptosis. This occurs regardless of the length of treatment. Reversibility of the p53-dependent response is determined by the length of chemotherapeutic treatment and correlates with specific changes in p53 target gene expression. Thus, transient chemotherapeutic treatments can exploit the differences between wild-type p53- and mutant p53-expressing prostate tumor cells.

It is proposed that the use of transient treatment protocols can exploit the reversibility of the p53-dependent checkpoint and justify the re-examination of the use of p53 status in clinical decision-making in prostate cancer. Further, the majority of primary prostate tumors express a wild-type p53. The role of p53 in promoting prostate tumor cell survival may explain the apparent selective pressure to retain wild-type p53 function in most tumors. This may provide important insights that will lead to better prognosis and more effective therapies.

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Appendices
None.